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DEVELOPMENT OF DEXTROSE BASE FORMULATION OF *BACILLUS SUBTILIS* AND IT'S EVALUATION AS A BIOCONTROL AGENT AGAINST *FUSARIUM OXYSPORUM F. SP. RICINI*

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ABSTRACT

In the present study; B. subtilis NSPLKMB01 has been isolated from local soil of Jalgaon district, Maharashtra, India, identified as a B. subtilis strain NAIMCC-B-02000, by 16S rRNA gene sequencing and developed formulation, using simple sugar dextrose anhydrous, as a carrier. Then bio-efficacy of the formulation was tested in randomized block design, against castor wilt causing agents Fusarium oxysporum f. sp. ricini, in pot trial through seed treatment. The results of a pot trial revealed that, treatment (T5), dextrose base Bacillus subtilis NSPLKMB01, 10g per kg of seed, gave highest disease control which was (73.41%) followed by chemical fungicide, treatment (T6) thiram (70.08%) and treatment (T1) talc base formulation of Bacillus subtilis NSPLKMB01 (70.14%). Similarly, maximum seed germination also recorded in treatment (T5), which was (94.10%), followed by chemical fungicide thiram (T6) and talc base formulation of Bacillus subtilis (T1), 91.09% and 83.92%, respectively.

KEYWORDS: Bioefficacy, Bacillus subtilis, Castor, Fungicide, Dextrose & Fusarium oxysporum

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Original Article

INTRODUCTION

Castor (*Ricinus communis L.*) is an important crop and cultivated throughout the world, because of the commercial importance of its oil. The commonly reported diseases of castor are bacterial leaf spot, caused by *Xanthomonas ricini* (Anon., 1971), wilt caused by *Fusarium oxysporum* f. sp. *ricini*, root rot caused by *Macrophomina phaseolina*, gray rot caused by *Botrytis ricini* (Anjani, *et. al*., 2004) etc. *Fusarium* wilt of castor is the most important soil and seed borne disease, in India and it causes yield loss to the extent of 80-100% (Anjani, *et. al*., 2004). Genetic makeup of *Fusarium spp.* is highly variable and causes morphological changes in the environment, in which they grow (Nelson., *et.al.*, 1983). In many in vitro studies, we have demonstrated that, some fungicides restrict or prevent the growth of fungal pathogens (Marley and Gbenga, 2004), but no measures are fully effective, at controlling these diseases. In the recent years, the environmental contamination caused by excessive use of chemical pesticides, increased the interest in integrated pest management, where chemical pesticides are substituted by bio pesticides, to control plant pests and diseases.

Some of the similar antagonists include, *Bacillus subtilis*; *Trichoderma spp*., *Pseudomonas fluorescens* provide good control against soil born plant pathogens. *Bacillus subtilis* have several advantages as a biological control agent because, they can produce endospores, which are tolerant to heat and desiccation. *Bacillus spp.* have been extensively used against many soil-borne plant pathogens, including *Rhizoctonia* (Yu *et al*., 2002) and

Fusarium (Schisler *et al*., 2002). It was reported that, the mechanism of this antifungal involves the production of antibiotics. Also, *B. subtilis* strain produces volatiles that, antagonizes are a range of soil borne plant pathogens. *B. subtilis* could secrete many antifungal metabolites, such as subtilin, bacitracin, bacillin, and bacillomycin, which have an inhibitory effect on fungal pathogens.

Anna Gunina and Yakov kuzyakov (2015), reported that, microorganisms rapidly uptake the sugar in the rhizosphere, for mobilization of nutrients. In the present study, formulation of *B. subtilis* NSPLKMB01 has been developed using simple sugar dextrose anhydrous, as a carrier. There are a number of carriers, which have been used for formulation development of bio-inoculants. Traditional carriers for bio agents are mostly talcum and liquids. There are some lacunas with existing formulations such as; the products are not completely soluble in water, high pH range, less shelf life of the products etc. The proposed new formulation, having the advantages like dextrose sugar act as a source of carbohydrate, for *B.subtilis* as well as rhizospheric microbes, when it goes into the field during application and long-term spore survival of *B.subtilis* is possible in the formulation, complete water solubility, neutral pH range and such formulations will be convenient for users, by drenching and spraying methods, than conventional ones. Similarly, in the present study; bio-efficacy of this formulation also tested against *F.oxysporum* f. sp. *ricini* and highest disease control were recorded, as compared to chemical fungicide and talc base formulation, of *B.subtilis* NSPLKMB01.

MATERIALS AND METHODS

Isolation of Bacterium *Bacillus subtilis*

Rhizosphere soil from castor field was collected from the farm. Then, 10 g soil sample was taken in 3 replications and serially diluted with sterile distilled water, from 10^{-1} to 10^{-4} dilutions and 100 µl of each dilution was placed on nutrient agar plates. Nutrient agar plates then incubated for 48 hrs at 28°C. After incubation many types of bacterial colonies observed on plates. Out of them, well-isolated wrinkle, dry, irregular colonies were selected and transfer to hicrome bacillus agar plates of Himedia, for further confirmation of isolates. After 72 hrs of incubation yellowish green colonies were observed, on hicrome *Bacillus* agar plates. Similarly, the isolated bacterium was identified, based on colony characteristics, gram staining methods and by various biochemical tests, as given in Bergey's Manual (1984), of Determinative Bacteriology. Further characterization of the isolate was also done, using 16S rRNA gene sequencing.

Isolation of Pathogenic Fungi *Fusarium oxysporum*

Fusarium oxysporum was isolated from infected roots of castor, on potato dextrose agar (PDA) and purified by singled spore isolation technique. Isolated *Fusarium* was identified using primary and secondary characteristics, according to the Nelson *et. al.* (1983). Followed by isolation and identification, a spore of *Fusarium oxysporum* inoculated in potato dextrose broth (PDB) and incubated up to 10 days at 120 RPM, for inoculum preparation.

Strain Revival and Inoculum Preparation

Bacillus subtilis NSPLKMB01, which was isolated and identified, was used for the experiment. The culture was maintained on Luria-Bertani (LB) slant at 28 ± 2^0 C. Bacterial cells of *Bacillus subtilis* NSPLKMB01 were inoculated in difco sporulation broth and incubated upto 60 hrs at 120 RPM, on a rotary shaker.

Mass Multiplication and Formulation Development of *Bacillus subtilis* NSPLKMB01

Mass multiplication of *Bacillus subtilis* NSPLKMB01 was made in shake flasks, using a difco sporulation

medium. The five-liter flasks containing two-liter medium were inoculated, by 60 hrs old vegetative inoculums. The vegetative inoculums were developed from standard culture slants. Three replications were maintained. All flasks were incubated upto 60 hrs at room temperature, on a rotary shaker at 120 rpm. Followed by fermentation, the broth was centrifuged at 15000 RPM, for 15 minutes. Cells were harvested and subsequently, CFU of harvested cells was determined and prepared talc base, as well as dextrose base formulations.

BIO-EFFICACY OF DEXTROSE BASE BACILLUS SUBTILIS NSPLKMB01

Pot Trial

The pot trial was conducted at the R&D farm of Nirmal Seeds Pvt. Ltd. in Kharif- 2015, with an objective to test the efficacy of dextrose base formulations of *Bacillus subtilis* NSPLKMB01, against *Fusarium* wilt of castor in randomized block design. The inoculum of *Fusarium oxysporum* was prepared on a rotary shaker and achieved CFU $1x10^6$/ml, after 10 days of incubation at 120rpm. Then, fungal culture was incorporated into the sterilized soil in the proportion of 1:10 w/w, and filled with the sterilized pots. The pots were watered and kept for a week for the uniform spread of the pathogen. Before conducting the trial, pots were checked and confirmed for wilt incidence. The pots were used to test the efficacy of *Bacillus subtilis*, against *Fusarium* wilt of castor.

Table 1: Bio Efficacy of Bacillus subtilis NSPLKMB01 against Fusarium oxysporum f. sp. ricini

Tr. No	Treatments	Seed Germination* %	Wilt* incidence %	Wilt* control %
T1	Seed treatment with talc base *Bacillus subtilis* at 10 g/kg seed (control)	83.92 (57.04)	18.81 (10.83)	70.14 (44.54)
T2	Seed treatment with dextrose base *Bacillus subtilis* at 1 g/kg seed	69.44 (43.97)	36.01 (21.10)	42.80 (25.34)
T3	Seed treatment with dextrose base *Bacillus subtilis* at 3 g/kg seed	72.29 (46.29)	33.77 (19.74)	46.35 (27.61)
T4	Seed treatment with dextrose base *Bacillus subtilis* at 5 g/kg seed	73.35 (47.17)	33.12 (19.34)	47.40 (28.29)
T5	Seed treatment with dextrose base *Bacillus subtilis* at 10 g/kg seed	94.10 (70.21)	16.72 (9.62)	73.41 (47.23)
T6	Seed treatment with Thiram at 2 g/kg seed	91.09 (65.68)	18.54 (10.68)	70.8 (45.06)
T7	Control (Untreated)	50.81 (30.23)	62.96 (39.01)	0
	SE ±	0.53	0.14	0.26
	CD (P=0.05)	1.63	0.45	0.79

*Mean of three replications
Figures in parentheses are arcsine transformed values.

RESULTS

Effect on Germination

The result presented in “Table 1” indicated that, all the treatments exhibited significantly higher seed germination, over untreated controls. However, the seed treatment with dextrose based *Bacillus subtilis* at 10g per kg seed (T5) recorded significantly, the highest seed germination (94.10%). This was followed by the seed treatment (T6), chemical fungicide thiram at 2g per kg seed (91.09%) and seed treatment (T1) talc base *Bacillus subtilis*, at 10g per kg seed recorded (83.92%) seed germination. The rest of the treatments also recorded maximum seed germination (69.44 to 73.35%), as against significantly, least seed germination (50.81%) in the untreated control.

Effect on Wilt Incidence and Disease Control

The data "Table 1" also revealed that, all the treatments recorded significantly minimum wilt incidence and maximum disease control, as compared to the treatment of control (untreated). However, the seed treatment with dextrose based *Bacillus subtilis* at 10g per kg seed (T5), recorded significantly least wilt incidence (16.72%) and the highest wilt control (73.41%). This was followed by the seed treatment (T6) thiram, at 2g per kg seed (18.54% & 70.80%) seed treatment (T1) talc base *Bacillus subtilis*, at 10g per kg seed (18.81% & 70.14%), with wilt incidence and wilt control, respectively. The rest of the treatments also recorded comparatively minimum wilt incidence (33.12 to 36.01%) and maximum wilt control (42.80 to 47.40%), as against significantly, the highest wilt incidence (62.96%) in the untreated control.

DISCUSSIONS

The adaptation of sustainable agricultural practices, gaining worldwide recognition. Application of bio control agents, for the management of insect pest, is one of the key elements of such sustainable agricultural practices. The genus *ricinus* is nonspecific, but its evolution under natural selection in a wide range of agro-climatic areas, has resulted in numerous wild and semi-wild types, with wide genotypic and phenotypic diversity. India is one of the centers of origin, wilt caused by *Fusarium oxysporum* f. sp *ricini*, is the most important soil and seed-borne disease of castor, in India. Biological control of soil borne plant pathogens is the realistic approach. In the present study, formulations of *B. subtilis* NSPLKMB01 have been developed using dextrose sugar, as a carrier and tested for germination, as well as bio efficacy against *Fusarium oxysporum* f. sp *ricini* of castor, at various doses. Dextrose anhydrous is colorless, odorless, white crystalline powder, having less than 0.5% moisture mostly used in foods, beverages, and pharmaceuticals, but in the present attempt dextrose anhydrous sugar used as a carrier, for the formulation of *B. subtilis* NSPLKMB01 and the highest percentage of germination, with maximum *Fusarium* wilt control was recorded.

Effect on Germination

Biological agents are reported for improvement of germination, in various crops. I Kutet and Cokorda Javandira (2016), reported that *Bacillus spp*. improved the germination of Tomato seedling. V. Shanmugaiah *et.al* (2009) and P. Lalitha *et.al* (2012), further reported that, application of *Pseudomonas fluorescens, Trichoderma viride* improved the germination in cotton and mustard crop. Results presented in "Table 1" also indicated that, formulation of *B. subtilis* NSPLKMB01 improved the germination of castor, as compared to chemical fungicide thiram and talc base formulation of the same strain of *B. subtilis*. The highest seed germination (94.10%) recorded at the dose 10g per kg of seed (T5), which was 10.18% higher, than talc base formulation of *B. subtilis* (T1) and 3.01% higher than chemical fungicide thiram (T6). At lower doses of formulation, i.e 1g, 3g & 5g per kg of seed, germination improved to 69.44%, 72.29% & 73.35%, respectively, which was also significantly superior, as compared to control 50.81%, but when does of formulation goes double i.e. 10g per kg of seed, the highest germination recorded, which was 94.10%. Might be the formulation of dextrose sugar, which was coated with castor seed 10g per kg of seed, attributed to accelerate the seed germination process, by improving the enzymatic activities and protein synthesis.

Effect on Wilt Incidence and Disease Control

Management of soil-borne fungal diseases is the most critical problem because; they survive years together in the soil and developed resistance, against most of the chemical fungicides. Excess application of chemicals causes health and

environmental problems. Therefore, alternative chemical measures are required. Antagonistic bacteria like *B. subtilis*, having multiple modes of action. Blakeman and Brodie (1977), postulated different mechanisms of disease control, that includes direct parasitism, production of extra cellular antibiotics, competition and stimulation of host defense. Madhusudhana Reddy Janga (2017), isolated various *Bacillus spp.* out of them, four isolates controlled the *Fusarium* wilt of castor to the extent of 65 to 70%. Similarly, H. B. Singh (2014), also reported that, *Trichoderma viride* and *Aspergillus niger* AN 27 control the wilt of Caster, which was caused by *Fusarium oxysporum* f.sp *ricini.* In the present study, it was observed that, all treatment were significantly superior, as compared to control. All formulations inhibited the growth of *Fusarium* and reduced the wilt significantly, but the most effective treatment was T5, where dextrose base formulation of *B. subtilis* NSPLKMB01 (10g per kg of seed), used for seed treatment, provided the highest reduction in *Fusarium* wilt of castor (73.41%) with minimum wilt incidence (16.72%), which was followed by chemical fungicide thiram and talc base formulation of *B. subtilis.* According to Raman Thangavelu (2010), there are multiple products of bio agents are available in the market, are mostly based on inert materials, which do not support the multiplication of microbes, when goes into the field. As per the results recorded in the present study, highest reduction observed in the *Fusarium* wilt of castor if dextrose sugar used as a carrier for *B. subtilis* NSPLKMB01, as compared to a talc base formulation of the same strain of *B. subtilis*, as well as chemical fungicide thiram. It indicated that, the carrier might have a certain role, when goes into the soil with antagonistic microbes. It might favor early germination and activation of *B.subtilis*, as well as native rhizospheric microorganism, which provided the highest reduction of *Fusarium* wilt, as compared to a talc base formulation of *B. subtilis.*

CONCLUSIONS

It could be concluded that, application of a dextrose base formulation of *B. subtilis* NSPLKMB01 increases the germination percentage of castor, and control the wilt caused by *Fusarium oxysporum* f.sp. *ricini*, significantly. Similarly, the current study provides strong evidence that, application of dextrose sugar as a carrier for biocontrol agents might be a promising alternative for farmers to obtain better yields with efficient and ecofriendly control of Phyto pathogens.

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